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# Apoptosis induced by death receptors

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**Abstract**

Death receptors belong to the TNF receptor family and are characterised by an intracellular death domain that serves to recruit adapter proteins such as TRADD and FADD and cysteine proteases such as Caspase-8. Activation of Caspase-8 on the aggregated receptor leads to apoptosis. Triggering of death receptors is mediated through the binding of specific ligands of the TNF family, which are homotrimeric type-2 membrane proteins displaying three receptor binding sites. There are various means of modulating the activation of death receptors. The status of the ligand (membrane-bound vs. soluble) is critical in the activation of Fas and of TRAIL receptors. Cleavage of membrane-bound FasL to a soluble form (sFasL) does not affect its ability to bind to Fas but drastically decreases its cytotoxic activity. Conversely, cross-linking epitope-tagged sFasL with anti-tag antibodies to mimic membrane-bound ligand results in a 1000-fold increase in cytotoxicity. This suggests that more than three Fas molecules need to be aggregated to efficiently signal apoptosis. Death receptors can also be regulated by decoy receptors. The cytotoxic ligand TRAIL interacts with five receptors, only two of which (TRAIL-R1 and -R2) have a death domain. TRAIL-R3 is anchored to the membrane by a glycolipid and acts as a dominant negative inhibitor of TRAIL-mediated apoptosis when overexpressed on TRAIL-sensitive cells. Intracellular proteins interacting with the apoptotic pathway are potential modulators of death receptors. FLIP resembles Caspase-8 in structure but lacks protease activity. It interacts with both FADD and Caspase-8 to inhibits the apoptotic signal of death receptors and, at the same time, can activate other signalling pathways such as that leading to NF-κB activation. 

*Keywords:* Apoptosis; Tumor necrosis factor (TNF); FLIP; Death receptors; FasL

## 1. Characteristics of apoptosis

Programmed cell death, also called apoptosis, is a physiologic event allowing a multicellular organism to smoothly get rid of old, superfluous, damaged or infected cells in a process which is clearly distinct from necrosis, the result of accidental cell death. Apoptosis is involved in a variety of processes such as the regression of defined tissues during development, the elimination of incompetent or self-reactive T-lymphocytes during positive and negative selection, or the elimination of non- or low-reactive B cells during affinity maturation. Cytotoxic T-lymphocytes, which are able to induce apoptosis in targets such as virus-infected cells, are eliminated by an apoptotic process called activation-induced cell death once the infection has been overcome. Tissues like the skin or the intestinal mucosa, which experience rapid turnover and regeneration, have sites where massive programmed cell death does occur. Apoptosis is also a favoured response in cells exposed to stress stimuli and damaging conditions, therefore preventing the appearance and proliferation of potentially dangerous cells. However, deregulation of the apoptotic process leading to either increased or reduced cell death can contribute to various pathologic conditions such as autoimmune diseases, AIDS or cancer.

At the morphological level, apoptosis is characterised by several of the following hallmarks: cell shrinking, chromatin condensation and cellular fragmentation into so-called apoptotic bodies. The latter are taken up by neighbouring or specialised cells, therefore avoiding the inflammatory response that would occur after spilling of intracellular material. These macroscopic changes are pre-

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ceded by biochemical events such as the redistribution of membrane lipids, the loss of mitochondrial membrane potential, the activation of intracellular proteases (caspases), the proteolytic degradation of selected proteins in the cell and the fragmentation of DNA at internucleosomal sites.

The central event in apoptotic cell death is the activation of caspases, a family of cysteine proteases with strict cleavage specificity at aspartic acid residues (Thornberry and Lazebink, 1998). Caspases pre-exist in the cell as inactive zymogen precursors composed of a prodomain followed by a large and a small catalytic subunits which, after proteolytic processing, fold into an active caspase able to specifically cleave a variety of cellular substrates, leading to the apoptotic phenotype.

There are two main pathways leading to the activation of effector caspases: the first one is triggered in response to a variety of stress conditions such as UV irradiation, growth factor deprivation, abnormal mitosis or anti-cancer drugs, and proceeds via the recruitment and activation of Caspase-9 into a complex containing cytochrome *c* and Apaf-1. Pro- and anti-apoptotic members of the Bcl-2 family, which act on the mitochondria, regulate this pathway (Green and Reed, 1998). The second pathway utilises specialised membrane receptors, which are efficiently coupled to caspase activation, especially Caspase-8 (Ashkenazi and Dixit, 1998). These death-inducing receptors belong to the tumour necrosis factor receptor (TNFR) family and are activated upon engagement by their cognate ligands of the TNF family.

## 2. The TNF and TNFR families of ligands and receptors

The TNF and TNFR families (Fig. 1) are mainly involved in the development and function of the immune system and of cells of lymphoid origin (Gravestein and Borst, 1998). For example, TNF plays a crucial role in inflammation, and the lymphotoxin system controls the development of peripheral lymphoid organs and splenic architecture. OX40 and 4-1BB play a co-stimulatory role in T cell proliferation whereas CD40 is a central player in B cell proliferation and immunoglobulin isotype switch. The RANK/OPG system modulates osteoclast maturation and bone homeostasis and the Fas/FasL system fulfils an important role in the homeostasis and in the cytotoxic effector function of T cells. A subset of the TNF receptors, namely TNF-R1, Fas, TRAMP/DR3, TRAIL-R1, TRAIL-R2 and DR6 (Fig. 1), can signal cell death via an intracellular "death domain" (DD), and is therefore termed the death receptor subfamily. TNF receptors are type-1 membrane proteins characterised by cysteine-rich sequences in their extracellular domains that fold into modules stacking one on top of each other thus conferring to the receptor an elongated structure (Fig. 1). Ligands of the TNF family are type-2 membrane proteins whose extracellular C-termini fold into β-sheet "jelly roll" structures that homotrimerise to form the active ligands. Ligands, which can exert their functions in the membrane-bound form or after proteolytic processing to a soluble trimer, can bind three receptors at their monomer boundaries (Banner et al., 1993). The intracellular domains of ligand-aggregated receptors can, in turn, bind to specific signalling molecules. Fas recruits the adaptor protein FADD via DD–DD interaction. FADD, in turn, binds Caspase-8 via homotypic interaction involving "death effector domains". TNF-R1 can also recruit Caspase-8 via TRADD and FADD and, in addition, potently activates the NF-κB transcription factor via the DD-containing kinase RIP and c-Jun N-terminal kinase via TRAF-2. Signalling through TRAMP/DR3 is similar to TNF-R1 (Ashkenazi and Dixit, 1998).

## 3. Modulation of death receptors activity

Apoptosis induced by death receptors can be controlled at several levels. Outside of the cell, the aggregation status of the ligand and the presence of soluble or membrane-bound decoy receptors have important regulatory roles. Anti-apoptotic intracellular proteins can block the apoptotic-signalling pathway or divert it into distinct responses. In addition, the overall sensitivity of a cell to apoptosis can be affected by engagement of receptors that do not themselves contain a death domain. The data presented below will exemplify some of these aspects.

### *3.1. Modulation of death receptors by ligands: the Fas/FasL system and other examples*

TNFα is synthesised as a membrane-bound protein that acts locally through cell to cell contact. Soluble TNFα (sTNFα) is released from the cell surface as the result of metalloproteinases cleavage (McGeehan et al., 1994; Mohler et al., 1994). Although both cell surface and secreted TNFα appear to be biologically active, deleterious physiological responses such as cachexia and endotoxic shock are mediated by the later. FasL is also processed and shed from the surface of human cells. Until recently, it was not clear whether the activities described for FasL were primarily due to its cell surface or secreted form. Elevated levels of sFasL were found in sera from patients with large granular lymphocytic leukemias and natural killer cell lymphomas (Tanaka et al., 1996). Since the administration of recombinant FasL or agonistic Fas antibodies into mice leads to liver failure and to rapid death of the animals (Ogasawara et al., 1993; Rensing-Ehl et al., 1995), it has been proposed that sFasL is implicated in the pathogenesis of various diseases such as hepatitis or AIDS.

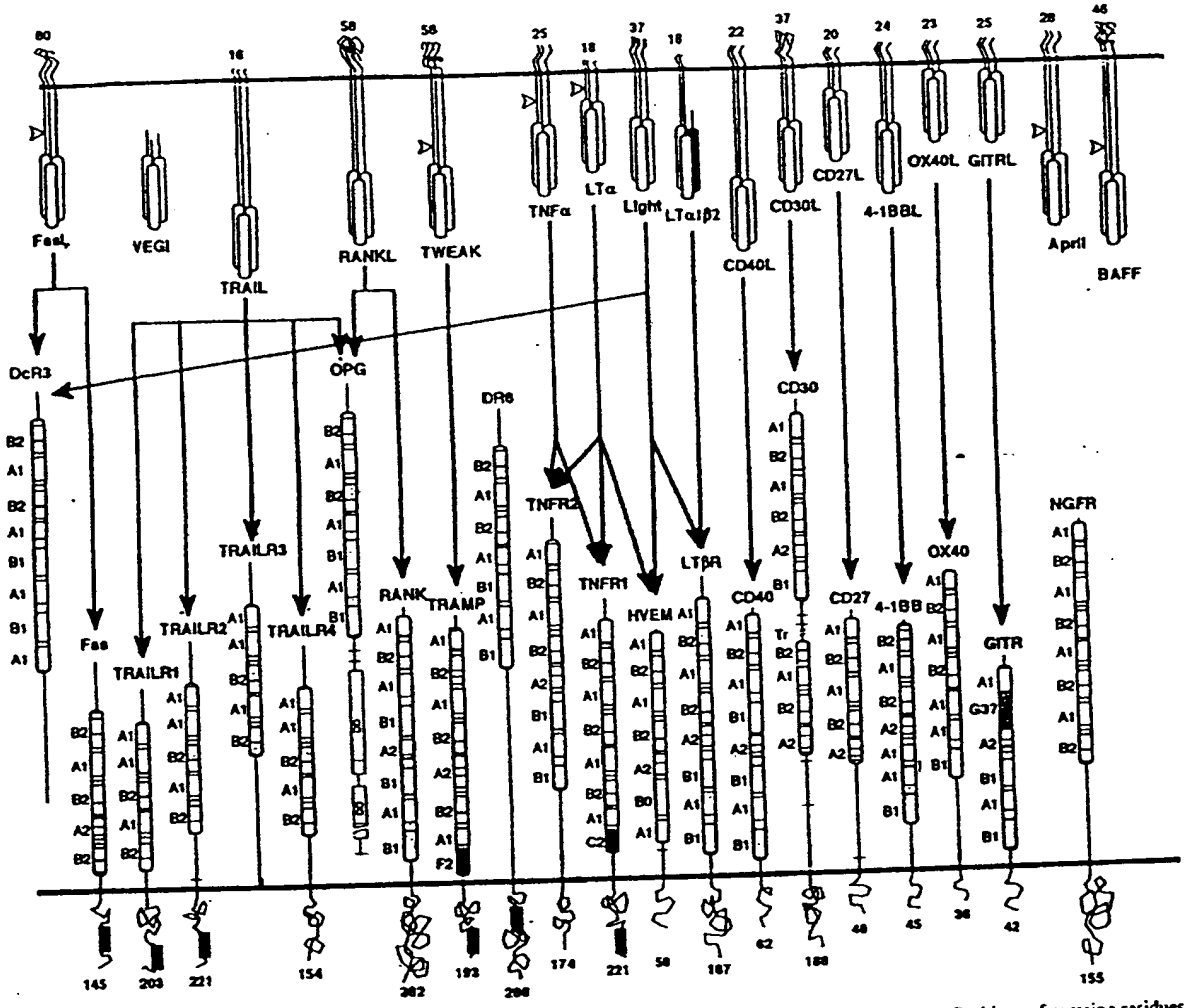


Fig. 1. TNF and TNFR family members. Trimeric ligands are shown at the top and receptors at the bottom of the figure. Positions of cysteine residues in the extracellular portion of receptors are indicated by horizontal bars; Basic structural modules (e.g., A1, A2, B1, B2) (Naismith and Sprang, 1998) are shown as shaded areas. The length of the intracellular domains is indicated by numbers. When present, death domains are represented by black boxes. Interactions that have been reported in the literature are indicated by arrows. The soluble receptor OPG contains two death domain motives in its C-terminal portion. Some of the ligands and receptors have several names: FasL/Apo-1L/CD95L, VEGI/TL-1, TRAIL/Apo-2L, RANK/OPGL/TRANCE, TWEAK/Apo-3L, GITRL/AITRL, BAFF/TALL-1/THANK/BlyS, DcR3/TR6, Fas/Apo-1/CD95, TRAILR1/DR4, TRAILR2/DR5/Killer/Apo-2, TRAILR3/DcR1, TRAILR4/DcR2, TARMP/Apo-3/DR3/WSL-1/LARD, HVEM/ATAR, GITR/AITR.

293 cells transfected with FasL contained predominantly the unprocessed form of FasL, but a significant proportion of processed, soluble FasL was also recovered into the medium. Processing of FasL was mediated by a metalloprotease and occurred between $Ser^{126}$ and $Leu^{127}$, in the stalk linking the trimerisation domain of FasL to its transmembrane segment. As predicted, site directed mutagenesis of these residues to glutamic acid residues almost completely abolished FasL processing.

Both naturally processed FasL and Flag-tagged, recombinant soluble FasL bound to recombinant Fas and to the surface of Fas positive cells. Surprisingly, however, these soluble FasLs were almost completely unable to induce cell death into A20 or Jurkat cells, which are otherwise very sensitive to agonistic anti-Fas antibodies. These results suggested that the mere trimerisation of Fas by sFasL was not sufficient to efficiently trigger the death signal. Indeed, when we took advantage of the Flag tag (an octapeptide epitope recognised by a monoclonal antibody) to cross-link FasL, we observed a 1000-fold increase of FasL cytotoxic activity on a variety of cell lines and on primary mouse hepatocytes. A similar effect was observed when sFasL was administered to mice: the animals showed no sign of sickness if sFasL or cross-linking antibodies were injected alone, but died within 3 h when sFasL injection was followed by that of cross-linking anti-Flag

antibodies (Schneider et al., 1998). Massive apoptosis was detected in the liver of these mice, as observed in mice treated with agonistic anti-Fas antibodies (Ogasawara et al., 1993). This suggests that a cluster of several Fas trimers is required to signal apoptosis, and that cross-linking of soluble FasL trimers may mimic the membrane-bound form of FasL.

Local cell-to-cell contact appears to be critical for T cell specific immunosurveillance, and indeed, FasL-mediated killing of virus-infected or tumor target cells is a highly specific process which assures that neighbouring cells or tissues are not affected. This specificity can only be guaranteed if the cytotoxic ligand remains associated with the lymphocytes. Considering the hepatic toxicity of FasL, circulating sFasL, if active, would be pathological. However, patients with elevated levels of sFasL (Tanaka et al., 1996) do not suffer from hepatitis, implying that sFasL in vivo is at best poorly active, in agreement with our results.

We tested whether other members of the TNF family known to induce apoptosis also required cross-linking for their activity. Similar to FasL, sTRAIL was active at low concentrations only in the presence of cross-linking antibodies. In contrast, cross-linking of sTWEAK did not increase its cytotoxic activity on HT-29 cells, suggesting that this ligand can exert its apoptotic activity in the soluble form. Interestingly, the activity of sTNFα was differentially influenced by the presence of cross-linking antibodies, depending on the receptor triggered. Cross-linking only increased the action of TNFα on TNF-R2 but not on TNF-R1 (Schneider et al., 1998). This is in line with results demonstrating that membrane-bound TNFα is the prime activating ligand for TNF-R2-mediated responses (Grell et al., 1995). We also found that the proliferative effect of sCD40L on primary human B cells was considerably increased upon cross-linking.

Taken together, these results suggest that several receptors are efficiently stimulated only by membrane-bound or cross-linked ligands. This probably provides a threshold ensuring that the signal is only transmitted under defined conditions, such as a cell-to-cell contact.

### *3.2. Modulation of death receptors by decoy receptors: the example of TRAIL-R3*

The cytotoxic ligand TRAIL shows a broad tissue distribution (Wiley et al., 1995) and has the particularity to interact with five receptors (Fig. 1). Two of them (TRAIL-R1/DR4 and TRAIL-R2/DR5) contain a death domain and are cytotoxic. The other three may act as decoy receptors. TRAIL-R3/DcR1 and TRAIL-R4/DcR2 are membrane-bound but lack a functional death domain, whereas OPG is a soluble receptor with dual specificity for TRAIL and RANKL/OPGL. In contrast to FasL, TRAIL is not cytotoxic to tissues despite the wide expression of TRAIL-R1 and -R2, suggesting that TRAIL receptor signalling is under the control of strong regulatory mechanisms.

TRAIL-R3 cDNA encodes a short receptor, which is devoid of an intracellular domain and ends immediately after a carboxy-terminal stretch of 15 hydrophobic amino acids. This feature is reminiscent of a signal for the addition of a glycosyl-phosphatidylinositol (GPI) anchor and the sequence indeed fulfils all structural requirements for GPI addition at $Ala^{236}$ (Udenfriend and Kodukula, 1995). In mature GPI-anchored proteins, the carboxy-terminal portion of the protein is exchanged for a preformed glycolipid, which anchors the protein to the membrane.

Several lines of evidence indicate that TRAIL-R3 is indeed a GPI-anchored protein. First, TRAIL-R3 expressed in 293 cells partitioned quantitatively into the detergent phase of a Triton X-114 phase separation, as expected for a GPI-anchored protein. Second, TRAIL-R3 was biosynthetically labelled with [$^3$H]inositol, which, in a protein, is highly specific of a GPI. The third evidence comes from the radiochemical analysis of TRAIL-R3. The hallmark of GPI-anchors is a non-acetylated glucosamine that can be deaminated with nitrous acid and reduced with sodium-borotritide to yield a radioactive anhydromannitol residue, with concomitant loss of the phosphatidylinositol. Subsequent dephosphorylation with hydrofluoric acid releases the neutral carbohydrate fraction of the GPI which, in the case of TRAIL-R3, co-migrated with authentic $Man_3$-anhydromannitol, the minimal conserved carbohydrate structure present in all GPI anchors analysed so far. Taken together, these data demonstrate the presence of a GPI in TRAIL-R3. Finally, a number of GPI-anchored proteins can be solubilised by the action of phosphatidylinositol-specific phospholipase C (PI-PLC). This reaction is, however, not possible if the hydroxyl group on position 2 of the inositol is acylated. This is most likely the case for TRAIL-R3 expressed in 293 cells, because it was resistant to the action of PI-PLC.

Because TRAIL-R3 can bind to TRAIL, yet is unable to transduce a death signal, it was expected that it could counteract the action of TRAIL in sensitive cells either by scavenging TRAIL or by forming inactive mixed receptors with TRAIL-R1/R2. MCF-7 cells respond to TRAIL or TNF by stopping proliferation and displaying morphological features of apoptotic cells. However, MCF-7 cells stably transfected with a dominant negative version of FADD became entirely resistant to the effects of TRAIL, TNF and agonistic anti-TRAIL-R2 antibodies, indicating that FADD-DN efficiently interacts with death signalling as previously described (Chinnaiyan et al., 1996; Schneider et al., 1997). MCF-7 cells stably transfected with TRAIL-R3 bound approximately 20 times more TRAIL on their surface than non-transfected cells, but only became susceptible to TRAIL with 100 times greater doses than those necessary to kill wild type cells. These TRAIL-R3 transfectants, however, still had functional apoptotic sig-

nalling pathways as demonstrated by their unaltered susceptibility to TNF and agonistic anti-TRAIL-R2 antibodies. These results point to the fact that TRAIL-R3 can indeed act as a dominant negative inhibitor of TRAIL action. Whether this reflects the role of TRAIL-R3 in vivo remains to be determined. However, experiments performed on melanoma cell lines revealed no correlation between TRAIL-R3 expression and resistance to TRAIL (Griffith et al., 1999; Zhang et al., 1999). Clearly, intracellular regulators of the pathway also play an essential role. In addition, Northern blot analysis indicated that TRAIL-R3 expression is less widespread than those of TRAIL-R1 and TRAIL-R2, suggesting that it may play a role in a restricted number of cell types.

### *3.3. Modulation of death receptors by other receptors: the example of TWEAK*

The widely expressed ligand TWEAK can induce apoptosis in a restricted number of cell lines (Chicheportiche et al., 1997). It also induces angiogenesis and proliferation of endothelial cells (Lynch et al., 1999). We found that TWEAK binds to the rhabdomyosarcoma Kym-1 cell line and efficiently kills it. This cytotoxicity could be prevented with either blocking anti-TNF antibodies, or with soluble TNF-R1 or with antagonistic anti-TNF-R1 antibodies, suggesting that the action of TWEAK was indirect and mediated by the induction of endogenous TNF. When Kym-1 cells were treated plus or minus TWEAK in the presence of blocking anti-TNF antibodies, cells survived and no difference was observed. However, TWEAK-treated cells were efficiently killed by sublethal doses of agonistic anti-TNF-R1 antibodies. This synergism indicates that TWEAK receptor is able to enhance TNF-R1-mediated apoptosis in Kym-1 cells. TWEAK is therefore not cytotoxic on its own in this model system but only helps to provide factors allowing for autocrine/paracrine killing of the cells via TNF and TNF-R1 (Schneider et al., 1999). Sensitization of cells to TNFα can also occur upon stimulation of TNF-R2, CD30 or CD40, pointing to the fact that cross-talk between receptor may be a general mechanism (Grell et al., 1999).

### *3.4. Modulation of death receptors by intracellular factors: the example of FLIP*

Caspase-8 is recruited to FADD via interaction between death effector domains. We found that ORF E8 of the equine herpes virus-2 encoded a protein with two predicted death effector domains. This protein, called v-FLIP, is also present in several other viruses including human herpes virus-8. It interferes with the apoptotic pathway of FasL by binding to FADD and potently inhibits TRAIL-mediated cell death (Thome et al., 1997). The sequence information from v-FLIP led to the discovery of a mammalian homologue, cellular FLIP (c-FLIP, also called CASPER/I-FLICE/Flame/CASH/CLARP/MRIT or usurpin) which is expressed in several splice variants (Tschopp et al., 1998). The longer form of c-FLIP (c-FLIP/long) contains two death effector domains and an additional caspase-like domain with significant homology to Caspase-8 (and Caspase-10). In contrast to Caspase-8, however, c-FLIP/long lacks essential features that are required for substrate catalysis and is therefore devoid of proteolytic activity. c-FLIP/long binds to both FADD and Caspase-8 via its death effector domains. The caspase-like domain also binds to Caspase-8, suggesting that the intimate contact occurring between c-FLIP and Caspase-8 may interfere with the activity of the latter. Indeed, both c-FLIP/short and c-FLIP/long inhibited cell death induced by several death receptors. c-FLIP/long appeared to be a better inhibitor than c-FLIP/short and both were especially effective against TRAIL-mediated apoptosis (Irmler et al., 1997). It is predicted that c-FLIP blocks apoptosis at the level of Caspase-8 by preventing the activation of downstream effectors such as Caspase-3. FLIP and other viral or cellular proteins involved in the intracellular regulation of death pathways, such as the IAPs, CrmA and Bcl-2 family members may contribute to the resistance of death receptor expressing cells to the action of death ligands (Tschopp et al., 1998). This could be the case of T cells during the early phase of activation, which are resistant to FasL despite abundant Fas expression. Expression of FLIP in various systems efficiently activates NF-kB transcription factor and the MAP kinase ERK pathway, suggesting that its physiological role may be more than just blocking apoptosis.

## 4. Conclusions

The activation of apoptosis via death receptors is a tightly regulated event. Soluble or membrane-bound decoy receptors can interfere with the death pathway by either scavenging the death ligand or by forming inactive mixed receptor complexes, thereby acting as dominant negative inhibitors.

The aggregation state of the ligand is probably a crucial factor. Some receptors can efficiently signal cell death only if they are sufficiently aggregated in a complex comprising more than three receptors. This requirement counteracts undesired activation of the receptor by systemic soluble trimeric ligands but still allows specific activation of the receptor during a cell-to-cell contact.

Preliminary experiments indicate that the efficiency of apoptosis signalling through TNF-R1 can be increased upon engagement of others receptors not directly linked to apoptosis. Further experiments are needed to determine if this is an exceptional or a more general mechanism.

Finally, anti-apoptotic intracellular proteins that interfere with the apoptotic machinery can dictate the outcome of death receptor triggering.

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